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Diagnosis of Honey Bee Diseases

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Abstract

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Apiary inspectors and beekeepers must be able to recognize bee diseases and parasites and to differentiate the serious diseases from the less important ones. This handbook describes laboratory techniques used to diagnose diseases and other abnormalities of the honey bee and to identify parasites and pests of the honey bee. Emphasis is placed on the techniques used by the U.S. Department of Agriculture Bee Research Laboratory. Included are directions for submitting, through APHIS-PPQ or State regulators, samples of suspected Africanized honey bees for identification of subspecies. Also included are directions for sending diseased brood and adult honey bees for diagnosis of bee disease.

Keywords: honey bee disease, honey bee disorder, honey bee parasite, honey bee pest, Africanized honey bee

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Diagnosis of Honey Bee Diseases

Hachiro Shimanuki[1] and David A. Knox

Introduction

Inspection for bee disease is an important part of beekeeping. Apiary inspectors and beekeepers must be able to recognize bee diseases and parasites and to differentiate the serious diseases from the less important ones. The purpose of this publication is to acquaint readers with laboratory techniques used to diagnose diseases and to detect and identify parasites, pests, and other abnormalities of the honey bee. We realize that different laboratory methods are used by others; where possible, those methods are described. However, emphasis is placed on the techniques used by the U.S. Department of Agriculture Bee Research Laboratory. Directions for submitting samples for diagnosis or subspecies identification are included in appendixes A and B.

Methods of Diagnosing Disease

Techniques of Microscopic Examination

Most bee diseases can be diagnosed by observing the associated microorganisms with a light microscope. The following techniques are commonly used to prepare microscope slides for examination:

Modified Hanging Drop

The modified hanging drop technique (Michael 1957) can be very useful for differentiating diseases of the brood. Residue from a suspected cell is first mixed with water. Then a drop of this suspension (smear) is placed on a cover glass. The suspension used should always be dilute and only slightly turbid. The smear is dried and fixed under a heat lamp, or the smear can be air dried and heat fixed by passing it rapidly through a bunsen burner flame two or three times. The fixed smear is stained with carbol fuchsin[2] or a suitable spore stain for 10 seconds. Enough stain should be placed on the cover glass to cover the entire smear. The excess

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[2] Solution A: 0.3 g basic fuchsin (90% dye content), 10 mL ethyl alcohol (95%); solution B: 5 g phenol, 95 mL distilled water. Mix solutions A and B.

stain is then washed off with water. While the smear is still wet, the cover glass is inverted with the smear side down and placed onto a standard microscope slide previously coated with a very thin layer of immersion oil. The slide is gently blotted dry and examined with a microscope using the oil immersion objective. Results: Organisms that are not heat fixed are caught in areas where pockets of water have formed in the oil, and the organisms usually exhibit Brownian movement (see section on American foulbrood).

Simple Stain

This method relies solely on differentiating the bacteria by morphology. Place a drop of the suspension directly on a microscope slide. Heat fix and stain the smear as described in the previous section. Carbol fuchsin, methylene blue, and safranin are examples of stains that can be used. Air-dry or gently blot the stained smear. Place a drop of immersion oil directly on the smear. No cover glass is necessary. Examine the slide, using the oil immersion objective. Results: Organisms are uniformly stained and easily distinguished.

Gram Stain

The Gram stain is a standard microbiological method that can be substituted for the simple stain. Briefly, the procedure is as follows: A fixed smear is stained with crystal violet, immersed in iodine solution, decolorized in ethyl alcohol, and counterstained with safranin. Results: Gram-positive organisms are blue; Gram-negative organisms are red.

Wet Mount

The wet mount is especially useful for examining fungi or protozoa. Macerate a portion of the sample in water. Place a drop of the suspension on a microscope slide, and carefully drop the cover glass on it to minimize air pockets. No stain is required. The wet mount is usually examined with the dry objectives of a microscope. Results: Organisms refract light and are therefore visible on the slide. A phase-contrast microscope may be helpful, especially if an oil immersion objective is required.

Microinjection Techniques

To diagnose some diseases or to determine toxic levels of sample materials, it may be necessary to feed or inoculate larvae, pupae, or adult honey bees. Michael (1960) developed a technique using a microinjector

equipped with a syringe and a 30-gauge needle. The microinjector can be calibrated to repeatedly deliver uniform inoculating volumes as small as 1 µL. The apparatus can be used to introduce material orally into the midgut (ventriculus) of a larva or to feed individual adult honey bees. The microinjection technique can also be adapted to direct injections into the body cavity of larvae, pupae, and adults.

Collecting Honey Bee Larvae and Pupae

Honey bee larvae 3 to 5 days old are readily obtained by removing a brood frame containing the desired larvae from a colony and placing it horizontally above a towel-lined tray in an incubator at 34°C. Within a few hours, the larvae crawl from their cells and drop to the tray below. Pupae can be easily obtained by collecting 5-day-old larvae as described above and incubating them in petri dishes until pupation occurs. This method of collecting larvae and pupae in large numbers saves considerable time and labor. It also eliminates the damage that can occur when attempting to remove these immature forms from cells by mechanical means.

Oral Introduction

Larvae

Honey bee larvae as young as 3 days and weighing as little as 25 mg can be force-fed by carefully inserting the needle through the mouthparts and into the esophagus (fig. 1). When the actuating lever is depressed, a

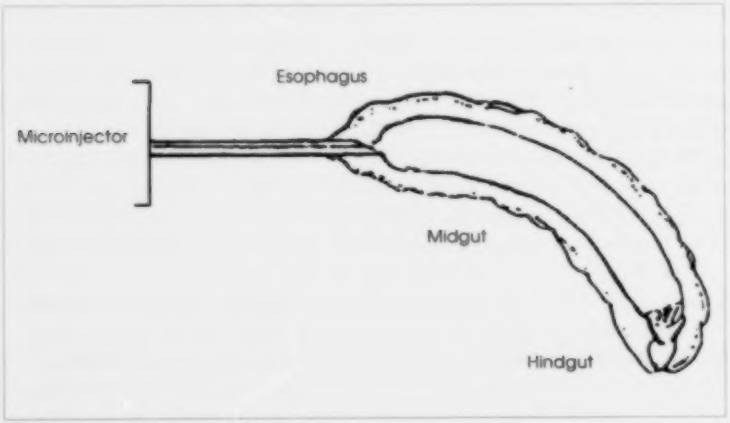


Figure 1. Force-feeding a honey bee larva.

predetermined volume of material is propelled through the esophagus and into the midgut, with no physical damage to the larva. After feeding, the larvae are placed in petri dishes lined with filter paper and incubated at 34°C.

Adults

Individual adult honey bees can also be fed known volumes using the microinjector. Adult bees are collected and held in a cage for about 4 hours without food. The material to be fed, at the final concentration, should be mixed into a sucrose solution to make it attractive to the bees. The microinjector is first actuated to produce a known volume of liquid at the tip of the needle. Then a bee from the cage is grasped by the wings, held up to the drop of liquid, and allowed to feed. Cold temperatures can be used to slow the bees and make them easier to handle. Avoid the use of carbon dioxide as an anesthetic to aid in handling bees, because they are reluctant to feed after exposure to carbon dioxide and their longevity is reduced. After feeding, the bees are placed in small cages with a supply of sugar syrup and held in an incubator at 34°C.

Direct Injection

Care should be taken to insert only the tip of the needle into the hemocoel and to not exceed inoculating volumes of 2 µL per bee.

Brood

Injections are usually confined to 4- to 5-day larvae. The larva is held gently between the first and second fingers and the thumb. and the larva must be absolutely parallel to the needle. After the integument is punctured by the needle under gentle pressure, the inoculum is expelled directly into the dorsal blood vessel; the needle is then withdrawn in a slow, steady movement. Any excessive pressure on the larva by the fingers, particularly when withdrawing the needle, must be avoided to prevent bleeding. Pupae can be inoculated dorsally between the third and fourth abdominal segments or through the propodeum of the thorax. After injection, the larvae or pupae are placed in petri dishes lined with filter paper and incubated at 34°C. If excessive bleeding has occurred, it can be seen on the filter paper.

Pupae can also be left in brood combs and inoculated in the head capsule (Wilson 1970). The pupal head is exposed by removing the cell cap, and the needle is inserted between the ocelli or through the clypeal sclerite.

Adults

Adult bees can be injected either through the propodeum of the thorax or dorsally through the intersegmental membrane between the third and fourth abdominal segments. Adult bees should be carefully subjected to carbon dioxide anesthetic before and during the process of injection. When bee longevity is a fa..tor in the test, cold temperature can be used as an anesthetic. After injection, the bees are placed in small cages with a supply of sugar syrup and held in an incubator at 34°C.

Removal of Digestive Tract

Intact digestive tracts that have been removed from adult honey bees are very useful for the detection of protozoan diseases (refer to fig. 9). The digestive tracts can be easily obtained by removing the head of the bee to free the digestive tract, grasping as much of the stinger as possible with a pair of fine tweezers, and then with a steady, gentle pull withdrawing the entire digestive tract. Freshly killed honey bees are required for this procedure.

Brood Diseases

Brood combs from healthy colonies typically have a solid and compact brood pattern. Almost every cell from the center of the comb outward contains an egg, larva, or pupa. The cappings are uniform in color and are convex (higher in center than at margins). The unfinished cappings of healthy brood may appear to have punctures, but since cells are always capped from the outer edges to the middle, the holes are always centered and have smooth edges. In contrast, brood combs from diseased colonies usually have a spotty brood pattern (pepperbox appearance), and the cappings tend to be darker, concave (sunken), and punctured. Also, the combs may contain the dried remains of larvae or pupae (scales), which are found lying lengthwise on the bottom side of brood cells. Sometimes scales are difficult to locate because of the condition of the comb. Scale material can be easily located using long-wave ultraviolet or near-ultraviolet light. Exposure to wavelengths of 3100-4000 angstroms will cause scale material to fluoresce. Some discretion must be used with this technique because honey and pollen may also fluoresce. Symptoms of various brood diseases are summarized in table 1.

Symptoms of a contagious disease are sometimes mimicked because of an unrelated factor. For instance, often brood that is neglected because of a shortage of nurse bees will die from either chilling or starvation.

Table 1. Comparative symptoms of various brood diseases of honey bees

Symptom	American foulbrood	European foulbrood	Sacbrood	Chalkbrood
Appearance of brood comb	Sealed brood. Discolored, sunken, or punctured cappings.	Unsealed brood. Some sealed brood in advanced cases with discolored, sunken, or punctured cappings.	Sealed brood. Scattered cells with punctured cappings, often with two holes.	Sealed and unsealed brood. Affected larvae usually on outer fringes.
Age of dead brood	***Usually older sealed larvae or young pupae.***[1] Upright in cells.	***Usually young unsealed larvae***; occasionally older sealed larvae. Typically in coiled stage.	Usually older sealed larvae; occasionally young unsealed larvae. Upright in cells.	Usually older larvae. Upright in cells.
Color of dead brood	Dull white, becoming light brown, coffee brown to dark brown, or almost black.	Dull white, becoming yellowish white to brown, dark brown, or almost black.	Grayish or straw-colored, becoming brown, grayish black, or black. Head end darker.	Chalk white. Sometimes mottled with black spots.
Consistency of dead brood	***Soft, becoming sticky to ropy.***	Watery; rarely sticky or ropy. Granular.	***Watery and granular; tough skin forms a sac.***	Watery to pastelike.
Odor of dead brood	Slight to pronounced glue odor to gluepot odor.	Slightly sour to penetratingly sour.	None to slightly sour.	Slight, non-objectionable.
Scale characteristics	Uniformly lies flat on lower side of cell. Adheres tightly to cell wall. Fine, threadlike tongue of dead pupae may be present. Head lies flat. Brittle. Black.	Usually twisted in cell. Does not adhere tightly to cell wall. Rubbery. Black.	Head prominently curled toward center of cell. Does not adhere tightly to cell wall. Rough texture. Brittle. Black.	***Does not adhere to cell wall.*** Brittle. Chalky white, mottled, or even black.

[1] Bold italics indicate the most useful field characteristics.

Symptoms can also be the result of a failing queen, laying workers, toxic chemicals, or poisonous plants. (See section on Noninfectious Diseases.)

Bacterial Diseases

American Foulbrood

Bacillus larvae is the bacterium that causes American foulbrood disease (AFB). *Bacillus larvae* is a slender rod with slightly rounded ends and a tendency to grow in chains (fig. 2). The rod varies greatly in length, from about 2.5 to 5 microns (µm), and is about 0.5 µm wide. The spore is oval and approximately twice as long as wide, about 0.6 by 1.3 µm. When stained with carbol fuchsin, the spore walls appear reddish purple and quite clear in the center. The spores may form clusters and appear to be stacked. Approximately 2.5 billion spores are produced in each infected larva. If the larva has been infected for less than 10 days, the vegetative cells are present, and some newly formed spores may be seen.

The modified hanging drop technique can be very useful for differentiating American foulbrood from other brood diseases. In areas of the smear where pockets of water are formed in the oil, the spores of *Bacillus larvae* exhibit Brownian movement. This is an extremely valuable diagnostic technique because the spores formed by the other *Bacillus*

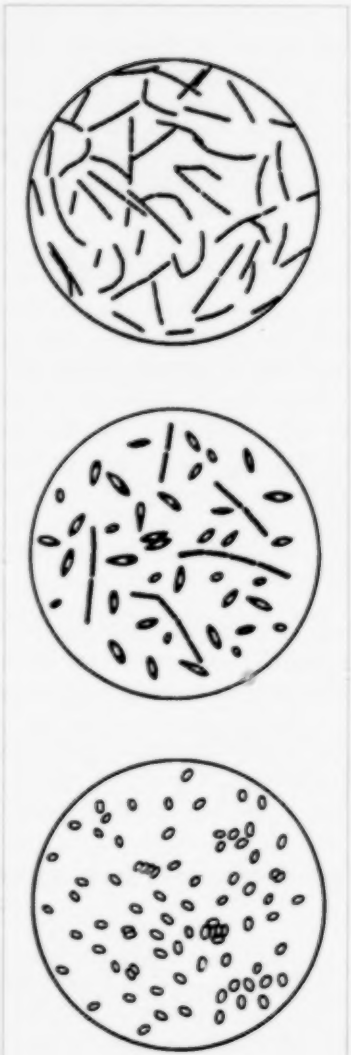


Figure 2. Bacterium that causes American foulbrood disease (not to scale): Top, *Bacillus larvae* vegetative cells; middle, *Bacillus larvae* spore formation; bottom, *Bacillus larvae* spores.

species associated with the known bee diseases usually remain fixed (see table 2). It is important to note that Brownian movement can be affected by slide preparation; also, debris and other bacteria can exhibit this motion. Therefore, Brownian movement must not be used as the sole criterion for diagnosis but must be considered together with the characteristic morphology of the spores and the gross larval symptoms. If microscopic examination is not conclusive, cultural tests can be made using the same suspension.

Cultivation of *Bacillus larvae*

Thiamine (vitamin B_1) and some amino acids are required for the growth of *Bacillus larvae*. Routine culture media such as nutrient broth will not support the growth of this organism. Good vegetative growth occurs on Difco brain heart infusion fortified with 0.1 mg thiamine hydrochloride per liter of medium (BHIT) and adjusted to pH 6.6 with HCl, but sporulation does not occur. Satisfactory growth and sporulation occur on the yeast extract, soluble starch, and glucose media recommended by Bailey and Lee (1962). The medium can be liquid, semisolid (0.3% agar), or solid (2% agar). For more information on sporulation, see Dingman and Stahly (1983).

Bacillus larvae spores also reproduce in the hemolymph of honey bee larvae, pupae, and adults when artificially introduced by injection (Michael 1960, Wilson and Rothenbuhler 1968, Wilson 1970).

To culture *Bacillus larvae*, we prepare spore suspensions by mixing diseased material (scales) with 9 mL sterile water in screw-cap tubes. (We use cotton swab applicators to remove and transfer the scales from

Table 2. Differentiation of *Bacillus* species in honey bees

Species	Brownian movement[1]	Catalase production	Nitrate reduction	Growth on nutrient agar
Bacillus larvae	+	–	+	–
Bacillus alvei	–	+	–	+
Bacillus laterosporus	–	+	+	+
Bacillus pulvifaciens	–	–	+	+

[1] In modified hanging drop technique.

the comb to the tubes.) The suspension is heat shocked at 80°C for 10 minutes (effective time) to kill nonsporeforming bacteria. A sterile cotton swab is used to evenly spread a portion of the suspension (approximately 0.2 mL) over the surface of BHIT agar plates, which are then incubated for 72 hours at 34°C. Individual colonies are small (1-2 mm) and opaque; however, if large numbers of viable *B. larva* spores are inoculated, a solid layer of growth will cover the plate.

There are no reliable methods for making plate counts of *Bacillus larvae* because fewer than 10 percent of the spores will produce visible growth on the media presently available (Shimanuki 1963). By calibrating our methods using spore and plate counts, we have determined that a minimum of 100 *B. larvae* spores are required to produce visible growth on BHIT.

Diagnostic Tests for *Bacillus larvae*

Holst Milk Test. The Holst milk test (Holst 1946) is a simple test based on the fact that a high level of proteolytic enzymes is produced by sporulating *Bacillus larvae*. The test is conducted by suspending a suspect scale or a smear of a diseased larva in a tube containing 3-4 mL of 1% powdered skim milk in water. The tube is then incubated at 37°C. If *B. larvae* is present, the suspension should clear in 10-20 minutes. It should be noted that this test is not always reliable.

Nitrate Reduction. *Bacillus larvae* reduces nitrate to nitrite (Lochhead 1937). The nitrate reduction test can be performed on a medium such as BHIT, which contains potassium nitrate (1-2 mg/L of medium). After growth has occurred, the addition of a drop of sulfanilic acid-alpha-naphthol reagent produces a red color if nitrate has been reduced to nitrite. Diagnosis should not be based on this test alone but on this test along with larval gross symptoms, bacterial morphology, and growth characteristics of the bacterial colony.

Catalase Production. A drop of 3% hydrogen peroxide is placed on an actively growing culture on a solid medium. Most aerobic bacteria break down the peroxide to water and oxygen and produce a bubbly foam, but *Bacillus larvae* is almost always negative for this reaction (Haynes 1972).

Fluorescent Antibody. The fluorescent antibody technique requires the preparation of specific antibodies stained with a fluorescent dye. Rabbits are injected with pure cultures of *Bacillus larvae*, and the active antiserum is collected and stained with a fluorescent dye. This fluorescent antiserum

is mixed with a bacterial smear on a slide and allowed to react. The excess antiserum is washed off the slide, and the slide is then examined with a fluorescence microscope. *B. larvae* appears as brightly fluorescing bodies on a dark background (Toschkov et al. 1970, Zhavnenko 1971, Otte 1973, Peng and Peng 1979).

Viability Test for *Bacillus larvae*

One method of controlling AFB is to destroy the viability of *Bacillus larvae* spores in contaminated bee equipment. This can be accomplished by gamma or electron beam irradiation or by fumigation with a sterilant gas such as ethylene oxide. Assessment of the efficacy of these methods should be based on the number of spores remaining viable in a test sample of brood comb containing at least 10 AFB scales.

A spore suspension is prepared from the sample comb by mixing 10 scales in 10 mL sterile water. Since each scale contains about 2.5 billion spores (Sturtevant 1932), 1 mL of the suspension should contain 2.5 billion spores. A portion of the suspension (0.2 mL = 500 million spores) is spread onto solid BHIT plates as previously described and incubated for 72-96 hours.

The results of the viability tests are reported as the approximate number of viable spores on a per-scale basis. If no colonies form on the medium, the results are recorded as <100 viable spores per scale; 1-9 colonies are recorded as <1,000 per scale; 10-99, <10,000 per scale; over 100 colonies per plate, >10,000 viable spores per scale; and when the plate is completely overgrown with colonies, the results are reported as no detectable reduction of viable spores.

Terramycin (Oxytetracycline) Resistance Tests

Isolates of *Bacillus larvae* can be screened for sensitivity to oxytetracycline based on the size of inhibition zones on agar plates. A spore suspension of the *B. larvae* isolate to be tested is spread on solid BHIT as described previously. A disk (BBL Sensi-Disc) containing 5 µg oxytetracycline is then placed on the plate, and the plate is incubated at 34°C for 72 hours. The zones formed by sensitive strains usually average 50 mm in diameter. Alternatively, oxytetracycline incorporated into liquid BHIT will inhibit the growth of sensitive strains of *B. larvae* at concentrations as low as 12 µg/L of medium (Gochnauer 1953). Care is necessary in making these tests, and adequate control strains should be included.

Any substantial reduction of the zone size or an increase in the concentration of oxytetracycline required to prevent growth of *Bacillus larvae* in liquid medium would be evidence of the development of resistant strains (Gochnauer et al. 1975). However, when interpreting the results of the tests, the effects of growth rates should be considered. Because strains often grow at different rates, one may falsely conclude that a strain is resistant. No resistance of *B. larvae* to oxytetracycline has yet been reported.

Detection of *Bacillus larvae* Spores in Hive Products

Honey. Occasionally it may be necessary to examine honey for the presence of *Bacillus larvae*. Due to the high concentration of carbohydrate and other natural bacteriostatic substance(s) in honey, the examination of honey requires special considerations. The classical method (Sturtevant 1932, 1936) is to dilute the honey 1:9 with water, centrifuge the mixture to concentrate the spores in the sediment, and then examine the sediment microscopically for the presence of spores.

We (Shimanuki and Knox 1988) have developed the following technique to detect *Bacillus larvae* spores in honey. Honey to be examined is heated to 45°C to permit easier handling and to decrease viscosity for more uniform distribution of any spores that may be present. Twenty-five milliliters of honey is placed in a 50-mL beaker and diluted with 10 mL of sterile water. The diluted honey is then transferred into a 1.75-inch (44-mm) dialysis tube. The open end is tied after the tube is filled, and the tube is submerged in running water for 18 hours or in a water bath with three to four water changes in that period. Following dialysis, the contents of the tube are centrifuged at about 2,000 g for 20 minutes. The supernatant is carefully removed with a pipet to leave approximately 1 mL of residue. This residue is resuspended in 9 mL of water in a screw-cap vial and heat shocked at 80°C for 10 minutes to kill nonsporeforming bacteria. Next, 0.5 mL of the suspension is spread onto a plate of BHIT agar. The plate is incubated at 37°C for 72 hours and examined for colonies of *B. larvae*. Difficulties can sometimes occur when honey samples contain other sporeforming bacteria that may completely cover the surface of the plate.

Since approximately 100 *Bacillus larvae* spores are required to produce visible growth on BHIT, this technique can demonstrate the presence of *B. larvae* spores in samples that contain a minimum of 80 spores per mL of undiluted honey (25 mL honey X 80 spores/mL = 2,000 spores/dialysis = 2,000 spores in 10 mL or 200 spores/mL; 0.5 mL = a 100-spore inocu-

lum). Lower spore levels can possibly be detected by the use of larger honey samples or a second centrifugation to further concentrate the spores.

Pollen. *Bacillus larvae* spores can also be recovered from bee-collected pollen pellets by physically removing bits of AFB scale. A series of sieves of different sizes is helpful. If scales are not detected, one may pass a water-pollen suspension through No. 2 filter paper, centrifuge the filtrate, and culture the pellet as described above (Gochnauer and Corner 1974).

Beeswax. We have had some success in recovering spores morphologically similar to those of *Bacillus larvae* by melting beeswax in boiling water, removing the beeswax cake after cooling, and centrifuging the water at 2,000 g for 20 minutes. The sediment is then examined microscopically for the presence of spores. Spores have also been recovered from contaminated beeswax by chloroform extraction (Kostecki 1969). However, in both cases, positive identification of the spores is not possible because the recovery techniques render the spores nonviable.

European Foulbrood

Melissococcus pluton (= *Streptococcus pluton*) is the bacterium that causes European foulbrood disease (EFB). *Melissococcus pluton* has been reclassified into the new genus *Melissococcus* (Bailey and Collins 1982a and b). However, *Melissococcus pluton* has still not been adequately described to be accepted for the current editions of "Bergey's Manual of Determinative Bacteriology."

Melissococcus pluton is generally observed early in the infection cycle before the appearance of the varied microflora associated with this disease. The *M. pluton* cell is short, nonsporeforming, and lancet shaped. The cell measures 0.5-0.7 by 1.0 µm and occurs singly, in pairs, or in chains (fig. 3).

Cultivation of *Melissococcus pluton*

Melissococcus pluton can be isolated on a medium developed by Bailey (1959). The medium consists of 1 g yeast extract (Difco), 1 g glucose, 1.35 g potassium dihydrogen phosphate (KH_2PO_4), 1 g soluble starch, 2 g agar, and distilled water to make 100 mL; the pH is adjusted to 6.6 with potassium hydroxide (KOH) and the mixture is autoclaved at 10 lb per square inch (116°C) for 20 minutes. It has been found that the addition of cysteine (0.1 g per 100 mL) improves the multiplication of *M. pluton* (Bailey and Collins 1982b).

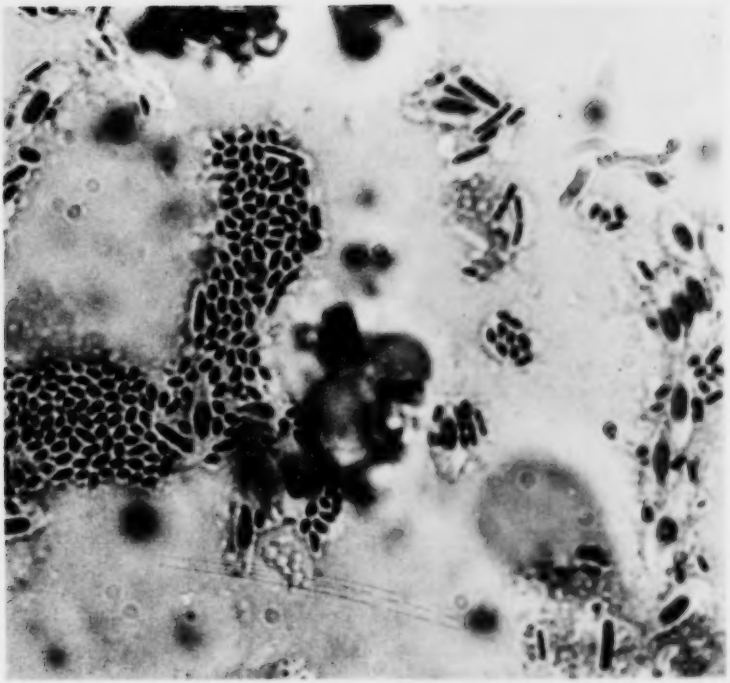


Figure 3. *Melissococcus pluton* and *Bacillus alvei* (X 1200).

It is difficult to isolate *Melissococcus pluton* on artificial media because of its growth requirements and the competition from other bacteria. Also, once isolated, identification of *M. pluton* is difficult due to its pleomorphic nature in culture (fig. 4). *Melissococcus pluton* is best isolated when few if any other organisms are present. According to Bailey (1959), it is best to dry smears of diseased larval midguts on a slide. A water suspension of this material or a suspension prepared from larvae (apparently healthy, infected, or dead), cappings, etc., can be streaked on freshly prepared Bailey's agar medium. Or decimal dilutions of these suspensions can be inoculated into molten Bailey's agar medium (45°C) and poured into plates (Bailey 1981). The plates are incubated anaerobically at 34°C. The "Gas Pak" (BBL) Anaerobic System, including a disposable hydrogen and carbon dioxide generator, is used to obtain anaerobic conditions. Small white colonies of *M. pluton* should appear after 4 days.

Figure 4. *Melissococcus pluton* as it appears in culture (X 1200).

Serodiagnosis

Pinnock and Featherstone (1984) developed an enzyme-linked immunosorbent assay (ELISA) for detecting *Melissococcus pluton*. Using this technique, they were able to demonstrate the presence of *M. pluton* even in apparently healthy honey bee colonies.

Organisms Associated With European Foulbrood

Some organisms do not cause European foulbrood, but they influence the odor and consistency of the dead brood and can be helpful in diagnosis. These secondary invaders include the following:

Bacillus alvei. The bacterium *Bacillus alvei* is frequently present in cases of European foulbrood disease (EFB). It is a rod 0.5-0.8 µm wide by 2.0-5.0 µm long (fig. 5). Spores measure 0.8 by 1.8-2.2 µm. Like *Bacillus larvae*, the spores may be clumped and appear stacked. The sporangium may be observed attached to the spore. Typical strains of *B. alvei* spread

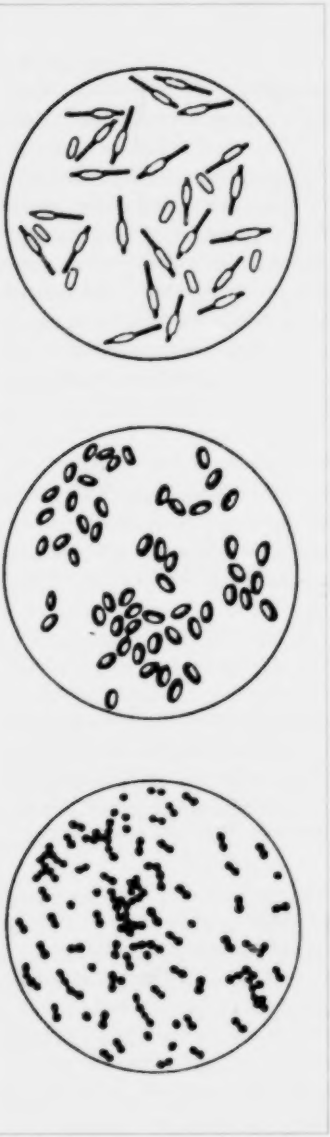


Figure 5. Bacteria associated with European foulbrood disease (not to scale): Top, *Bacillus alvei*; middle, *Bacillus laterosporus*; bottom, *Enterococcus faecalis*.

vigorously on nutrient agar and may show "motile colonies"; free spores may lie side by side in long rows on the agar. The growth of this bacterium produces an unpleasant odor.

***Bacillus laterosporus* (= *Bacillus orpheus*).** Rods of *Bacillus laterosporus* measure 0.5-0.8 µm by 2.0-5.0 µm, and the spores 1.0-1.3 by 1.2-1.5 µm (fig. 5). An important diagnostic feature is the production of a canoe-shaped parasporal body that stains very heavily along one side and the two ends, and remains firmly adherent to the spore after lysis of the sporangium. The clear portion with the finely outlined wall is the spore. *B. laterosporus* grows moderately on nutrient agar, becoming dull and opaque, and spreads actively if the agar surface is moist. Growth on nutrient agar with 1% glucose added (glucose agar) is thicker and may become wrinkled.

***Enterococcus faecalis* (= *Streptococcus faecalis* = *Streptococcus apis* = *Streptococcus liquefaciens*).** Ovoid cells (elongated in the direction of chain) are 0.5-1.0 µm in diameter and are usually in pairs or short chains (fig. 5). This organism resembles *Melissococcus pluton* and may exhibit Brownian movement when the modified hanging drop technique is used. Growth occurs on nutrient agar usually within 1 day. Colonies are generally smaller than 2 mm; they are smooth and

convex, with a well-defined border. When magnified, the colonies appear light brown and granular.

***Bacterium eurydice* (= *Achromobacter eurydice*).** There is no standard description of *Bacterium eurydice*. White (1912) described this organism as a small, slender Gram-negative rod with slightly rounded ends, occurring singly or in pairs and measuring 0.5-1.4 µm long by 0.4-0.7 µm wide. According to White (1920), *Bacterium eurydice* is best isolated by plating the midgut contents of infected larvae on glucose agar and incubating at room temperature. Growth is slow and never luxuriant, and colonies are convex, smooth, and glistening. However, later researchers, who were unable to isolate *B. eurydice* as described by White, used the name *Bacterium eurydice* for a Gram-positive bacterium isolated from diseased larvae. Therefore, mention of the name in the literature causes confusion. This organism is not included in the current editions of Bergey's manual.

***Bacillus apiarius*.** The bacterium *Bacillus apiarius* is rarely encountered and may or may not be legitimately associated with EFB. Rods are 0.6-0.8 µm in diameter and often less at the poles. Special diagnostic features include the ridged, thick, rectangular spore coat and the stainable remnants of the sporangium, which remain attached for a considerable time. Growth can occur on Sabouraud dextrose medium.

Powdery Scale

Bacillus pulvifaciens is the bacterium that causes powdery scale disease. Powdery scale disease is seldom reported, perhaps because the average beekeeper is unable to identify it. A useful diagnostic characteristic is the scale that results from the dead larva. The scale is light brown to yellow and extends from the base to the top of the cell. The scale is powdery; when handled, it crumbles into a dust.

Bacillus pulvifaciens vegetative cells measure 0.3-0.6 µm by 1.5-3.0 µm. The spores are 1.0 by 1.3-1.5 µm. The bacterium can be isolated on nutrient agar, but growth is heavier on glucose agar. When first isolated, the organism produces a reddish-brown pigment that can be lost by subculturing. *Bacillus pulvifaciens* closely resembles *Bacillus larvae*, but the spores do not exhibit Brownian movement in the modified hanging drop technique. Also, *B. pulvifaciens* is distinguished by its ability to grow at 20°C and by its growth on nutrient agar.

Fungal Diseases

Chalkbrood

Ascosphaera apis is the fungus that causes chalkbrood disease. *Ascosphaera apis* is a heterothallic organism and develops a characteristic spore cyst when opposite thallic strains (+ and –) fuse. Spore cysts measure 47-140 µm in diameter (fig. 6). Spore balls enclosed within the cyst are 9-19 µm in diameter, and individual spores are 3.0-4.0 µm by 1.4-2.0 µm.

Figure 6. Spore cyst of *Ascosphaera apis* containing spore balls, which in turn contain spores.

Chalkbrood disease can be easily identified by its gross symptoms. An affected larva becomes overgrown by fluffy cottonlike mycelia and swells to the size of the cell. If only one strain (+ or –) of mycelium is present, the larva dries into a hard, shrunken, white chalklike mummy—thus the name chalkbrood. When the + and – mycelia are present in a diseased larva, spore cysts can form, and the resulting mummies appear either mottled (black on white) or completely black. In heavily infected hives, mummies can be found at the hive entrances or on the bottom boards. Mummies can sometimes be detected in brood cells by tapping the comb against a solid surface. This easy removal of larval remains also differentiates chalkbrood from other brood diseases.

Ascosphaera apis grows luxuriantly on potato dextrose agar fortified with 4 g yeast extract/L. Growth and sporulation also occur on malt agar but less profusely and with no aerial hyphae: this facilitates subculturing and microscopic examination. Cultures have a characteristic fruity odor similar to that of fermenting peaches. The optimum temperature for growth is 30°C.

Ascosphaera apis can be easily isolated from newly infected larvae or fresh mummies. These can be placed directly on the medium and incubated. New mycelial growth is usually visible within 24 hours. Small blocks of agar containing mycelia can be transferred to new plates to obtain pure cultures and isolates of the + and – strains. *A. apis* can be

isolated from old mummies by placing them on water agar (agar with no added nutrients), incubating them, and transferring the new mycelial growth to a nutrient medium. Difficulties sometimes occur because *A. apis* may fail to grow or may be overgrown by other fungi, which can contaminate old mummies.

If only one strain (+ or –) is isolated, a fluffy cottonlike growth will eventually cover the plate. When both the + and – thalli are isolated, spore cysts form throughout the culture. The + and – thalli are morphologically identical. They can be distinguished by inoculating isolates on opposing sides of a plate. When opposite thalli grow together, a line of spore cysts forms at the juncture.

Stonebrood

Stonebrood is usually caused by *Aspergillus flavus*, occasionally *A. fumigatus*, and sometimes other *Aspergillus* species. These fungi are common soil inhabitants that are pathogenic to adult bees, other insects, mammals, and birds. The disease is difficult to identify in its early stages of infection. The fungus grows rapidly and forms a characteristic whitish-yellow collarlike ring near the head end of the infected larva. A wet mount prepared from the larva shows mycelia penetrating throughout the insect. After death, the infected larva becomes hardened and quite difficult to crush—hence the name stonebrood. Eventually, the fungus erupts from the integument of the insect and forms a false skin. At this stage, the larva may be covered with green powdery fungal spores. The spores of *Aspergillus flavus* are yellow green, and *A. fumigatus* spores are gray green. These spores can become so numerous that they fill the comb cells that contain the affected larvae.

Figure 7. Conidial heads of *Aspergillus flavus*.

Stonebrood can usually be diagnosed from gross symptoms, but positive identification of the fungus requires its cultivation in the laboratory and subsequent examination of its conidial heads (fig. 7). *Aspergillus* spp. can be grown on potato dextrose or Sabouraud dextrose agars.

Viral Disease: Sacbrood

Morator aetatulas is the virus that causes sacbrood disease. It is the only common brood disease that is caused by a virus. Since sacbrood-diseased larvae are relatively free from bacteria, laboratory verification is usually based on gross symptoms and the absence of bacteria. Positive diagnosis requires the use of a special antiserum. Affected larvae change from pearly white to gray and finally black. Death occurs when the larvae are upright, just before pupation. Consequently, affected larvae are usually found in capped cells. Head development of diseased larvae is typically retarded. The head region is usually darker than the rest of the body and may lean toward the center of the cell. When affected larvae are carefully removed from their cells, they appear to be a sac filled with water. Typically the scales are brittle but easy to remove. Sacbrood-diseased larvae have no characteristic odor.

Mixed Infections

Bacillus larvae produces a potent antibiotic that eliminates competition from other bacteria typically associated with honey bee larvae. For this reason, American foulbrood and European foulbrood are rarely found in the same colony, except in cases where AFB is just becoming established in colonies that already have EFB.

It is not unusual to find chalkbrood and sacbrood on the same comb or on a comb with larvae infected with AFB. However, no single larva has been found to be infected with more than one disease. This is an important point to remember when selecting a sample for disease diagnosis.

Diseases of Adult Bees

Most diseases of adult bees are difficult to diagnose because the gross symptoms are not unique. For instance, inability to fly, unhooked wings, and dysentery are general symptoms associated with many disorders. In most cases, microscopic examination is required for proper diagnosis.

Protozoan Diseases

Nosema

Nosema apis is the protozoan that causes nosema disease. *Nosema apis* spores are large, oval bodies, 4-6 µm long by 2-4 µm wide (fig. 8). The spores develop exclusively within the epithelial cells of the ventriculus of

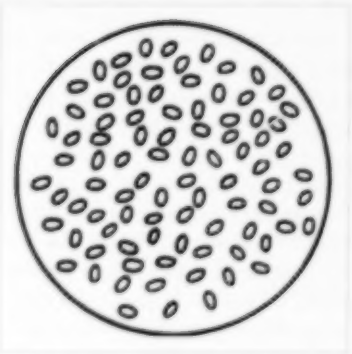


Figure 8. Nosema spores as they appear in a wet mount (X 400).

the adult honey bee. Nosema disease usually manifests itself in bees that are confined; therefore, the heaviest infections are found in winter bees, package bees, bees used for pollination in greenhouses, etc.

No single symptom typifies nosema disease. Differences between healthy bees and heavily infected bees can be seen by removing the digestive tract and examining the ventriculus. The ventriculus of a healthy bee is straw brown, and the individual circular constrictions are clearly seen (fig. 9). In a heavily infected bee, the ventriculus is white, soft, and swollen, obscuring the constrictions (White 1918). However, positive diagnosis can be made only by microscopic examination of suspect bees or their fecal material for the presence of *Nosema apis* spores. Samples of bees to be examined can be dried or preserved in alcohol. If the sample is partially decomposed, the presence of yeasts and molds resembling *N. apis* may make an accurate diagnosis difficult. For quick, routine examinations, the abdomens from 10 or more bees are removed, placed in a dish with 1.0 mL water per bee abdomen, and ground with a pestle or the rounded end of a clean test tube. A cleaner preparation can be obtained by grinding free digestive tracts. A wet

Figure 9. Top, Digestive tract from a healthy honey bee. Note the individual circular constrictions on the ventriculus. Bottom, Digestive tract of a honey bee with nosema disease.

mount is prepared from the resulting suspension and examined under the high dry objective of a compound microscope. Alternatively, individual bees can be examined to obtain an approximate percentage of infected bees in a colony. Also, a quantitative measure of levels of *Nosema* infection can be determined using a hemocytometer as described by Cantwell (1970).

Nosema can also be detected without sacrificing workers or queens, by examining their fecal material. A colony can be sampled by collecting feces of worker bees on glass plates near the hive entrance, scraping off a deposit, mixing it with water, and preparing a wet mount from the resulting suspension (Wilson and Ellis 1966). Suspect queens can be held in small petri dishes or in glass tubes and allowed to walk freely. They usually defecate within 1 hour. Queen feces appear as drops of clear, colorless liquid, which are then transferred to a microscope slide with a pipet or capillary tube. A cover glass is placed over the feces before examination with a high dry objective (L'Arrivee and Hrytsak 1964).

Amoeba

Malpighamoeba mellificae is the organism that causes amoeba disease. Since this protozoan is found in the Malpighian tubules of adult bees, diagnosis can be made only by the removal and microscopic examination of the tubules for the presence of amoeba cysts. The cysts measure 5-8 µm in diameter and can be seen in the infected Malpighian tubules (fig. 10).

The Malpighian tubules are long, threadlike projections originating at the junction of the midgut and the hindgut. The tubules can be teased away from the digestive tract with a pair of fine tweezers and then

Figure 10. Cross sections of Malpighian tubules. Top, Healthy tubule; bottom, tubule containing cysts of *Malpighamoeba mellificae*.

placed in a drop of water on a microscope slide. A cover glass is positioned over the tubules while applying uniform pressure to obtain a flat surface for microscopic examination. *Malpighamoeba mellificae* can be discerned using a high dry objective and then changing to the oil immersion objective for more detail.

Gregarines

Four gregarines (protozoans of the order Gregarinida) are associated with honey bees: *Monoica apis*, *Apigregarina stammeri*, *Acuta rousseaui*, and *Leidyana apis*. The immature stages, or cephalonts, average about 16 by 44 µm. Cephalonts are oval and consist of two distinct body segments; the posterior segment is larger. The mature stages, or sporonts, average about 35 by 85 µm and have a reduced anterior segment (fig. 11).

Gregarines are found attached to the epithelium of the midgut of adult honey bees. Gently remove the midgut from the digestive tract of a suspect bee and place it on a microscope slide in a drop of water. The midgut can be separated from the digestive tract at the point of attachment with the proventriculus (honey stomach) and hindgut using fine tweezers and a scalpel. Gently break open the midgut with fine tweezers and a probe, and place a cover glass over the resulting suspension. Gregarines can be seen using the low-power objective of a compound microscope.

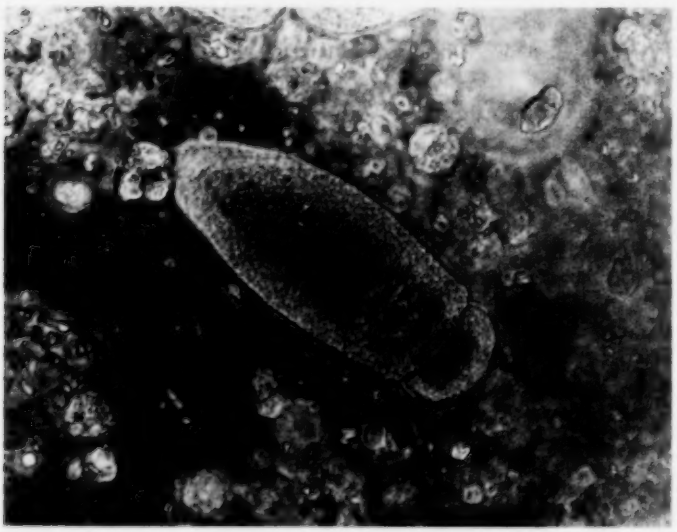


Figure 11. A gregarine sporont (mature stage).

Flagellates

Crithidia (= *Leptomonas*) species are the flagellates associated with honey bees. Flagellates have been found either free in the lumen or attached to the epithelium of the hindgut and rectum of adult honey bees (Fyg 1954). Flagellates vary in size from 5 to 30 µm. Some appear as pearlike bodies with flagella; others are long threadlike forms or are round without flagella (Lotmar 1946). To look for flagellates, remove the digestive tract of a suspect bee and place it on a microscope slide in a drop of water. Then, using fine tweezers and a scalpel, separate the hindgut and rectum at the point of attachment with the midgut. Macerate the hindgut and rectum, using a fine pair of tweezers and a probe. Place a cover glass on the resulting suspension and observe under the high dry objective of the microscope.

Bacterial Diseases

Septicemia

Pseudomonas aeruginosa (= *Pseudomonas apiseptica*) is the bacterium that causes septicemia in honey bees. This disease results in the destruction of connective tissues of the thorax, legs, wings, and antennae. Consequently, the affected bees fall apart when handled. Dead or dying bees may also have a putrid odor.

Pseudomonas aeruginosa rods measure 0.5-0.8 by 1.5-3.0 µm. They are Gram-negative and occur singly, in pairs, or in short chains. A bacterial smear and Gram stain can be easily prepared after removing a wing from the thorax and dipping the wing base in a drop of water on a microscope slide. To isolate this organism, streak the base of a wing across Difco *Pseudomonas* isolation agar or *Pseudomonas* Agar F. The optimum temperature for growth is 37°C. *Pseudomonas aeruginosa* in culture is characterized by the excretion of diffusible yellow-green pigments that fluoresce in ultraviolet light (wavelength below 260 nm).

Septicemia disease can also be diagnosed by reproducing the disease symptoms in healthy caged bees. This is accomplished by preparing a water extract (macerate the equivalent of one suspect bee per mL of water) and inoculating healthy bees through the thorax (see Methods) or dipping them in the water extract. Bees with septicemia die within 24 hours and exhibit the typical odor and the "break apart" symptom after approximately 48 hours.

Spiroplasmosis

Spiroplasma species is the bacterium that causes spiroplasmosis. *Spiroplasma* is a helical, motile, cell-wall-free prokaryote that is found in the hemolymph of infected adult honey bees. The organism is a tiny, coiled, and sometimes branched filament 0.7-1.2 µm in diameter (fig. 12). Its length increases with age and ranges from 2 to >10 µm (Clark 1977, 1978a).

Spiroplasma can be seen in the hemolymph using the oil immersion objective of a phase-contrast microscope. Hemolymph can be taken from adult bees by puncturing the intersegmental membrane directly behind the first coxae with a fine capillary tube made from the tip of a Pasteur pipet.

This organism can be cultured in standard mycoplasma broth medium (GIBCO) and in Singh's mosquito tissue culture medium with 20% fetal calf serum.

Viral Diseases

Chronic Bee Paralysis

Bees affected by chronic bee paralysis are usually found on the top bars of the combs. They appear to tremble uncontrollably and are unable to fly.

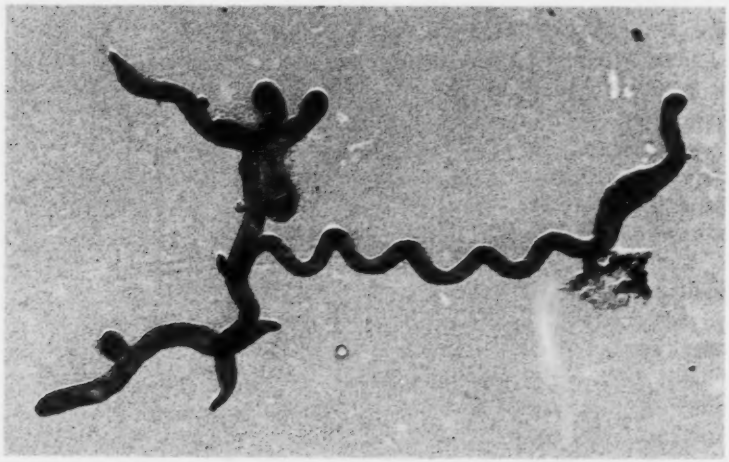


Figure 12. *Spiroplasma* species (X 21,840).

In severe cases, large numbers of bees can be found crawling out the hive entrance. Individual bees are frequently black, hairless, and shiny. However, in some cases, paralysislike symptoms can be caused by toxic chemicals.

Ideally, the diagnosis of paralysis disease is made using serological techniques. Since this is beyond the capability of most laboratories, diagnosis is usually made by observing symptoms in individual bees and, when possible, colony behavior.

Paralysis disease can be diagnosed by reproducing the disease symptoms in caged bees. This can be done by spraying, feeding, or injecting a water extract made from the suspect bees. The extract is prepared by macerating the equivalent of one suspect bee in 1 mL of water. It is then centrifuged to eliminate large suspended matter and passed through a 0.45-µm filter to remove bacteria. To feed up to 20 caged bees, mix 2 mL of the extract with an equal volume of sugar syrup. For inoculation, each bee receives 1 µL of the extract through a dorsal abdominal intersegmental membrane (see Methods). The symptoms of paralysis should be visible after 6 days. Appropriate control bees should be treated with extracts made from healthy bees.

Filamentous Virus

Filamentous virus is also known as F-virus and bee rickettsiosis. This disease, previously thought to be of rickettsial origin, can be diagnosed by examining the hemolymph of infected bees using phase-contrast microscopy. The hemolymph of honey bees infected with this virus is milky white and contains many spherical to rod-shaped viral particles of a size close to the limit of resolution for light microscopy. The viral particles consist of a folded nucleocapsid within a viral envelope (fig. 13) and are 0.4 by 0.1 µm (Clark 1978b).

Noninfectious Disorders

Noninfectious disorders can be the result of neglect, lethal genes, pollen or nectar from poisonous plants, toxic chemicals (pesticides), etc. Most often, dead or discolored pupae result from a noninfectious condition. For a good review of noninfectious diseases, see Tucker (1978).

Figure 13. Filamentous virus (X 67,250). Each viral particle consists of a nucleocapsid enclosed in a viral envelope.

Neglected Brood

Normally nurse bees feed and protect the brood. However, if there is a sudden shortage of adult bees, the larvae and pupae suffer and may die of chilling, overheating, or starvation.

Chilled Brood

Chilling usually occurs in early spring when brood nests expand rapidly and there is a shortage of adult bees to cover all the brood. Consequently, chilled brood is found most often on the fringes of the brood area and healthy brood at the center. However, chilling can also happen during cold weather following any sudden reduction of the worker bee population. Chilled larvae and pupae are often yellowish, tinged with black on segmental margins. They may also be brownish or black, crumbly, pasty, or watery. In extreme cases, brood cells are punctured and uncapped, and pupae are decapitated by the adult bees. It should be remembered that decapitation can also result from the larvae of the lesser wax moth.

Overheated Brood

The overheating of brood develops when there is a sudden loss of worker bees available to cool the colony during hot weather. Larvae that died

from overheating become brownish or black and are watery; pupae have a black, greasy appearance. Newly emerged adult bees may be wingless. Cappings of brood cells can appear melted, darkened, sunken, and punctured.

Starved Brood

Normally when there is shortage of food in a colony, larvae are removed and/or consumed by the adult bees. However, when there is a sudden shortage of adult bees available to feed the larvae, the larvae starve. Affected larvae are not restricted to the periphery of brood combs. The most striking feature of starved brood is the larvae crawling from the brood cells in search of food. Starved brood is almost always restricted to the larval stage. However, emerging bees may starve if they were stressed as pupae by chilling or overheating and if there are too few nurse bees to feed them soon after they have chewed through their cappings. These bees usually die with only their heads out of the cells and with their tongues extended.

Overheated Bees

Overheating in worker bees can occur when bees are confined in their hives during hot weather without proper ventilation or access to water. Bees dying from overheating crawl about rapidly while fanning their wings. They are often wet, and their wings appear hazy. In some cases, an abnormally large accumulation of dead bees may be seen at the hive entrance.

Genetic Lethality

Bees can also die from genetic faults during all stages of development, usually without exhibiting symptoms of known diseases. However, drone brood from laying workers and drone-laying queens often die with symptoms resembling EFB but in the absence of known pathogenic agents. Genetic lethality is the suspected cause of this condition.

Plant Poisoning

Poisonous plants can be a problem under certain conditions in limited areas. If a plant's nectar is poisonous, the symptoms of plant poisoning are limited to the blooming period of the plant. However, if the poison is in the pollen, the symptoms may linger as long as the pollen remains in the combs. There is no clear-cut method for differentiating between plant poisoning and pesticide poisoning. The effects of plant poisoning are

usually more gradual and last longer than the effects of pesticide poisoning. Plant poisoning usually occurs in the same geographical area at the same time each year, whereas pesticide poisoning is indiscriminate. For a good review of poisonous plants, see Barker (1978). Some examples of plant poisoning are listed below and in table 3.

Purple Brood

Purple brood occurs when adult bees collect and use the pollen and nectar from *Cyrilla racemiflora* (titi, southern leatherwood). This "disease" is characterized by the blue or purple color of the affected larvae.

Paralysis

Aesculus californica (California buckeye) is probably the best known of the poisonous plants in the United States. Field bees exhibit symptoms similar to those of chronic bee paralysis; i.e., the bees are black and shiny from loss of hair and they tremble. Also, either the eggs do not hatch or the larvae die soon after hatching.

Milkweed Pollinia

The pollen of milkweed (*Asclepias* species) is produced in pollinia (coherent pollen grains) that are attached in pairs by a slender filament. When removed from a flower, the pollinia resemble a wishbone with pollen masses hanging from the ends. Honey bees become ensnared in the thin pollinia attachment and free themselves by pulling the pollinia from the flower (fig. 14). Honey bees often become seriously encumbered and unable to effectively fly or crawl because of the structures that remain attached to their body parts.

Figure 14. Milkweed pollinia attached to honey bee.

Table 3. Comparative symptoms in honey bees poisoned by toxic chemicals and selected plants

Source of poison	Stages most affected	Effect on adult	Effect on brood	Effect on colony
Toxic chemicals	Adult	Field bees die in or near hive or in field. Nurse bees may also die. Queens usually not affected.	Usually few larvae killed. Symptoms of starvation may be evident if adult population reduced severely.	Weakened or killed. Many dead bees near hive.
California buckeye (*Aesculus californica*)	Young brood	Emerging young workers often deformed, pale. Some hairless and tremble. Queens lay eggs at reduced rate, cease, or become drone layers.	Eggs normal at outset; later fail to hatch or all are drone eggs. Larvae die soon after hatching and disappear. Little or no capped brood; if present, scattered.	Weakened or killed. May be many dead bees near entrance. Supersedure of queen may fail.
Yellow jessamine (*Gelsemium sempervirens*)	Larva, pupa, and young adult	Young workers affected and soon die. Old adults appear normal.	Pupae die in cells and become mummified.	Slightly to severely weakened.
Loco plants (*Astragalus* spp.)	Adult and pupa	Field bees die. Some become black and tremble. Queen may die.	Many cells contain dried pupae.	Population dwindles. Colony may die.
False hellebore (*Veratrum californicum*)	Adult	Many field bees die between plants and hive. Adults die in curled state. Queens not affected.	No effect.	Field population lost.
Southern leather-wood (*Cyrilla racemiflora*)	Larva	No effect.	Many blue or purple larvae. Larvae die in cells when nearly mature.	Slight to severe weakening.

Source: Modified from Burnside and Wansell (1936).

Pesticide Poisoning

The most apparent indication of serious pesticide poisoning is the sudden loss of adult bees. This loss is characterized by the appearance of many dead and dying adult bees and sometimes pupae at the colony entrances. However, in many instances, the bees are lost in the field before returning to the colony. If the pesticide is brought back to the hive by the foragers, the nurse bees are killed when they feed on contaminated honey or pollen and the brood will exhibit symptoms of neglect or poisoning. The symptoms of poisoned honey bees often depend on the class of pesticide involved (table 4).

A residue of a pesticide may be present as the original pesticide, or as an identifiable degradation product, or both. Frequently, the amount of residue is extremely small. Pesticide analysis consists primarily of the following (from Wilson et al. 1980):

(1) Blending and extraction of the biological material (such as bees or pollen) with a suitable solvent system to maximize the recovery of suspected pesticides and their metabolites. This eliminates the bulk of the biological substrate.

(2) A series of liquid-liquid extractions and column chromatographs to further separate the residues from other materials of biological origin. A very fast method for cleanup is gel permeation chromatography. Large molecules of biological origin emerge from the column first, thereby directly trimming the crude extract down to a much cleaner sample.

(3) Detection of residues at the highest possible sensitivity to avoid interference from substances not previously removed. The most popular detection system is gas-liquid chromatography, wherein the residue-containing sample is volatilized and chromatographed as a vapor. Thin-layer and paper chromatography are also useful for establishing the identity of pesticide residues. Visualization for detection usually involves spraying the chromatograms with a chemical that reacts with the pesticide or metabolite to produce a characteristic color. Other methods used for residue analysis include mass spectroscopy and fluorometric techniques. The residue must be capable of absorbing visible light or ultraviolet light. The intensity of the reemitted light is measured at some suitable wavelength and compared with standards.

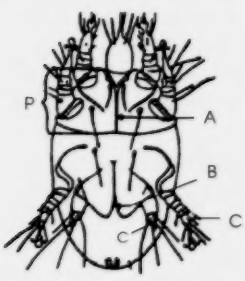
Table 4. Typical symptoms of pesticide poisoning

Class of pesticide	Examples of pesticides	Symptoms of poisoning
Organo-phosphorous	Parathion, methyl parathion, dimethoate (Cygon), naled (Dibrom), mevinphos (Phosdrin), diazinon, dichlorvos (Vapona), monocrotophos (Azodrin), malathion, tepp, phorate (Thimet), phosphamidon (Dimecron), chlorpyrifos (Dursban)	Regurgitation (bees are wet); disorientation, lethargy. Perhaps distended abdomen, erratic attempts to clean selves, tumbling about, paralysis, ultimately death. Wings held away from body but usually remaining hooked together. High percentage of poisoned bees die at colony.
Chlorinated hydrocarbon	Aldrin, chlordane, DDT, dieldrin, heptachlor, lindane, toxaphene	Erratic movements, abnormal activities, trembling. Hindlegs dragging as if paralyzed and wings held away from body but usually remaining hooked together. High percentage of poisoned bees die in field as well as at colony.
Carbamate	Carbaryl (Sevin), carbofuran (Furadan), aminocarb (Matacil), dimetilan (Dimetilane), mexacarbate, methomyl (Lannate, Nudrin)	Aggressiveness, erratic movements; then inability to fly, stupefaction as though chilled; followed by paralysis, morbidity, death. Most bees usually die at colony. Queens often cease egg laying; hive bees initiate supersedure, rearing queens before egg laying resumes.
Dinitrophenyl	Dinocap (Karathane), DNOC, DNOCHP, DNOSBP	Similar to symptoms of chlorinated hydrocarbons but often accompanied by regurgitation of substances in digestive tract, as is typical of organophosphorous pesticides. Most affected bees usually die at colony.
Botanical	Pyrethrum, allethrin, and the pyrethroids: resmethrin, bioresmethrin, bioethanomethrin, and cismethrin; also nicotine, rotenone, ryania, sabadilla	Perhaps regurgitation from highly toxic pyrethroids, together with erratic movements; then inability to fly and stupefaction, followed very soon by paralysis, moribundity, and death. Bees often die between foraging area and colony.

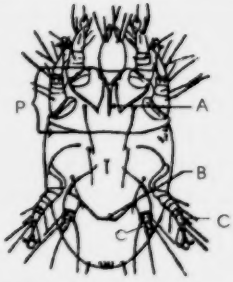
Source. Modified from Atkins (1975).

Parasitic Honey Bee Mites

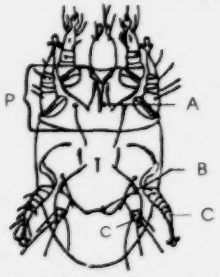
Three *Acarapis* species are associated with adult honey bees: *Acarapis woodi*, *A. externus*, and *A. dorsalis*. These mites are difficult to detect and identify because of their small size and similarity; therefore, they have frequently been identified by location on the bee instead of by morphological characters. Only *A. woodi* can be diagnosed solely on habitat; the position of other species on the host is a useful but not infallible characteristic. *Acarapis woodi* lives exclusively in the prothoracic tracheae; *A. externus* inhabits the membranous area between the posterior region of the head and thorax or the ventral neck region and the posterior tentorial pits. *Acarapis dorsalis* is usually found in the dorsal groove between the mesoscutum and mesocutellum and the wing bases. Morphological characters separating these species are shown in figure 15. Complete descriptions and illustrations are in Delfinado-Baker and Baker (1982).



Acarapis dorsalis: Apodeme (A) full length of propodosoma (P); coxal plate (B) deep indentation; tarsal joints (C) <10 µm.



Acarapis externus: Apodeme (A) 2/3 length of propodosoma (P); coxal plate (B) variable indentation; tarsal joints (C) >10 µm.



Acarapis woodi: Apodeme (A) 2/3 propodosoma (P); coxal plate (B) shallow indentation; tarsal joints (C) <10 µm.

Figure 15. Morphological characters separating *Acarapis* species.

Honey Bee Tracheal Mite (*Acarapis woodi*)

The female *Acarapis woodi*, or honey bee tracheal mite, is 143-174 µm long and the male 125-136 µm. The body is oval; widest between the second and third pair of legs; and whitish or pearly white with shining, smooth cuticle. A few long hairs are present on the body and legs. This mite has an elongate, beaklike gnathosoma with long, bladelike styles (mouthparts) for feeding on the host.

The population of *Acarapis woodi* may vary seasonally. During the period of maximum bee population, the number of bees with mites is reduced. The likelihood of detecting tracheal mites is highest in the fall. In sampling for this mite, one should try to collect either moribund bees that may be crawling near the hive entrance or bees at the entrance as they are leaving or returning to the hive. These bees should be placed in 70% ethyl or methyl alcohol as soon as they are collected. One should not collect bees that have been dead for an unknown period because they are less than ideal for the diagnosis of tracheal mites.

No one symptom characterizes this disease. An affected bee could have disjointed wings and be unable to fly, or have a distended abdomen, or both. Absence of these symptoms does not necessarily indicate freedom from mites. Positive diagnosis can be made only by microscopic examination of the tracheae; since only *Acarapis woodi* is found in the bee tracheae, this is an important diagnostic feature.

A healthy trachea appears cream color or white. The trachea of a severely infested bee has brown or black blotches with crustlike lesions and is obstructed by many mites in different stages of development (fig. 16). The trachea must be examined carefully for the presence of mites. The trachea may not always be discolored when mites are present, and a cloudy or discolored trachea does not always contain mites.

Methods for diagnosing *Acarapis woodi* are listed below. Each of these methods has its advantages and disadvantages.

Method 1

Pin the bee on its back and remove the head and first pair of legs by pushing them off with a scalpel or razor blade in a downward and forward motion (fig. 17). Using a dissecting microscope, remove the first ring of the thorax (tergite of prothorax) with forceps. This exposes the tracheal

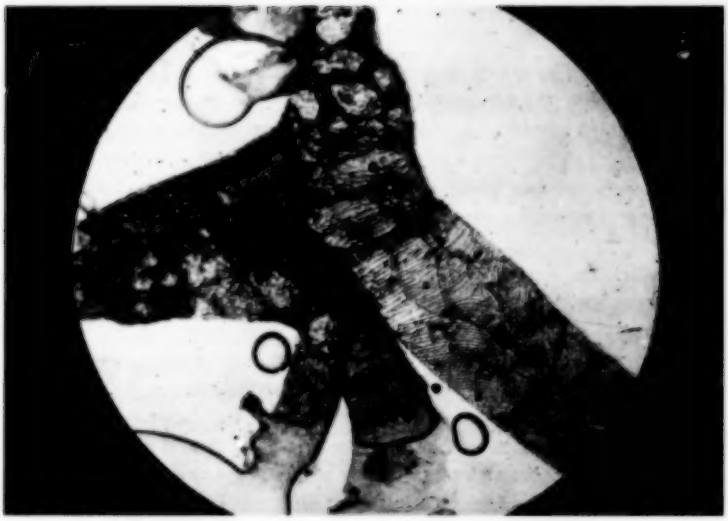


Figure 16. Trachea containing mites (X 40).

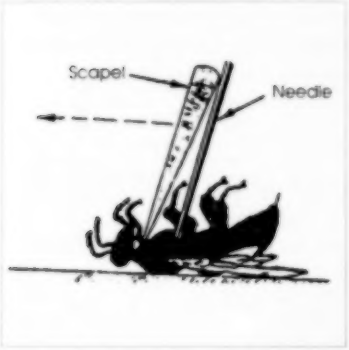


Figure 17. Positioning a bee for dissection.

trunks in the mesothorax (fig. 18). When the infestation is light, it is necessary to remove the trachea. Place the trachea in a drop of lactic acid on a glass slide for clearing, and cover with a cover glass for examination at X 40-100 on a compound microscope.

Method 2

Grasp the bee between your thumb and forefinger and remove the head and first pair of legs. Then with a scalpel, razor blade, or fine pair of scissors, cut a thin transverse section from the anterior face of the thorax in such a way as to obtain a disk. Place the disk on a microscope slide and add a few drops of lactic acid. This makes the material more transparent and also helps to separate the muscle. With the aid of a dissecting microscope, carefully separate the muscles, remove the trachea, and examine the preparations as in method 1. This procedure is recommended for quick examination of a few bees.

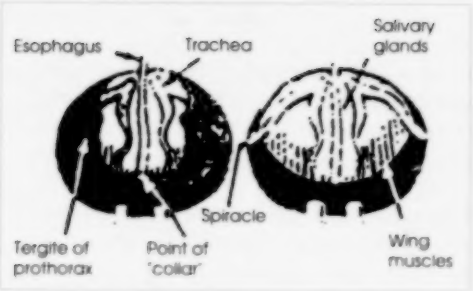


Figure 18. Location of the trachea in the thorax.

Method 3

Cut a few thoracic disks as described in method 2, place them on a slide, and add a few drops of 10% potassium hydroxide (KOH). Heat the slide gently for 1-2 minutes (do not boil), cover with a cover glass, crush the disks lightly, and examine microscopically. This procedure is advantageous when the bees have been dead for some time.

Method 4

Prepare transverse-section disks from the thoraces of 50 honey bees as described in method 2, place them in 5% KOH, and incubate at 37°C for 16-24 hours. The KOH dissolves the muscle and fat tissue, leaving the trachea exposed. Then examine the disk-trachea suspension under a dissecting microscope. Remove suspicious tracheae from the disks and examine the tracheae microscopically (X 40-100). This procedure is recommended for large samples of bees.

Method 5

Remove the heads, abdomens, wings, and legs from 20-200 thoraces and place them in a homogenizing jar with 25 mL of water. Homogenize three times for several seconds at 10,000 rpm, using just enough water to rinse the inside of the jar. Then strain the suspension through a 0.8-mesh sieve and rinse with water. The final volume of the filtrate should be about 50 mL. Centrifuge the filtrate at about 1,500 g for 5 minutes and discard the supernatant. Then add a few drops of lactic acid to the preparation, and allow it to stand for 10 minutes. Finally, place the sediment on a slide for examination. In this method, a microscope with an oil immersion objective is required to correctly identify *Acarapis woodi* because other mites associated with honey bees are morphologically similar. This technique is described by Colin et al. (1979).

Method 6

In the flotation method (Camazine 1985), bees cannot be stored or killed in alcohol. For cleanest preparations, remove the head, wings, legs, and abdomen (saving only the thoraxes) of recently killed bees. This removal is easily done using one's fingers when the bees are frozen. Place 25-100 bees in a household blender with enough water added to cover the blades. Blend the preparation for no more than 15 seconds, just until the thoraces are broken apart. (Blending for longer periods will pulverize the tracheae.) Pour the resulting mixture into a series of test tubes (2-3 cm in diameter). Most of the denser muscle fibers and cuticular fragments fall to the bottom while the tracheae and air sacs float, forming a thin whitish layer on the surface of the water. Suction off this layer with a pipette, place on one or more slides, and cover with cover slips. Examine the slides under a compound microscope at X 100-250. Examine the slide in a systematic manner for darkened, blotchy, and discolored tracheae and for undamaged tracheae that may also contain mites and eggs.

Method 7

In the modified methylene blue staining technique (Peng and Nasr 1985), prepare transverse-section disks from the thoraces of 50 bees as described in method 2. Place the disks in a beaker of 8% KOH solution, and heat to boiling with continuous gentle stirring of the disks. Remove the solution from the heat and continue stirring until the soft tissues inside the disks are dissolved and cleared (about 10 minutes). Excessive stirring and heating will damage the specimens and subsequently reduce the color intensity of the mites. Recover the disks from the KOH by filtration through a perforated Tissue-Tek processing capsule. After filtration, cover the processing capsule with a lid, place in a beaker, and wash with tap water to remove the remaining KOH. After washing, transfer the processing capsule to a modified methylene blue staining solution (prepared by first dissolving 1% aqueous methylene blue and then adding sodium chloride to make a 0.85% sodium chloride solution). Immerse the capsule in that solution for 5 minutes and then in distilled water for 2-5 minutes; finally, rinse the capsule with 70% ethyl alcohol. Examine the disks for stained mites within the tracheae under a dissecting microscope at X 10-25.

Method 8

Differentiation of live mites from dead mites (Eischen et al. 1986) is the method of choice for evaluating chemicals used to control tracheal mites.

Anesthetize live bees with carbon dioxide and remove the abdomens with a scalpel to prevent being stung during examination. Remove the head and first pair of legs of each bee by holding the bee on its back and gently pushing this section off with a downward and forward motion. Place each bee, held in this position, under a dissecting microscope, and remove the first ring of the thorax with fine forceps. This exposes the tracheal attachment to the thoracic wall, which is often the only location of mites in a light infestation. Remove tracheae that appear abnormal with tweezers and transfer to a glass slide containing a thin film of glycerol. Then dissect the tracheae using a pair of fine needle probes. Mites are considered dead if they do not move; also, dead mites often appear discolored and desiccated. Living mites have a translucent gray or pearl color and move within a few seconds after dissection.

Method 9

For serodiagnosis, Ragsdale and Furgala (1987) produced an antiserum against extracts of *Acarapis woodi*-infested tracheae to be used as the primary antibody in a direct enzyme-linked immunosorbent assay (ELISA). Ragsdale and Kjer (1989) improved the ELISA technique, making it as reliable as dissection for the detection of *A. woodi*. Their ELISA is accurate, sensitive, reproducible, cost effective, rapid, and easy to use.

Varroa jacobsoni

The mite *Varroa jacobsoni* can be found on adult bees, on the brood, and in hive debris. The most severe parasitism occurs on the older larvae and pupae, with drone brood being preferred to worker brood (Ritter and Ruttner 1980). In heavy infestations, pupae may not develop into adult bees. The adults that do emerge may have shortened abdomens, misshapen wings, and deformed legs and may weigh less than healthy bees (De Jong et al. 1982b).

The adult female mite is oval and flat, about 1.1 mm long and 1.5 mm wide, and pale to reddish brown; it can easily be seen with the unaided eye. The mites attach to the adult bee between the abdominal segments or between body regions (head, thorax, abdomen) and are therefore difficult to detect. However, they can be easily recognized against the white surface of pupae. Male mites are considerably smaller and are pale to light tan (Delfinado-Baker 1984). The life cycle of *V. jacobsoni* is summarized in figure 19.

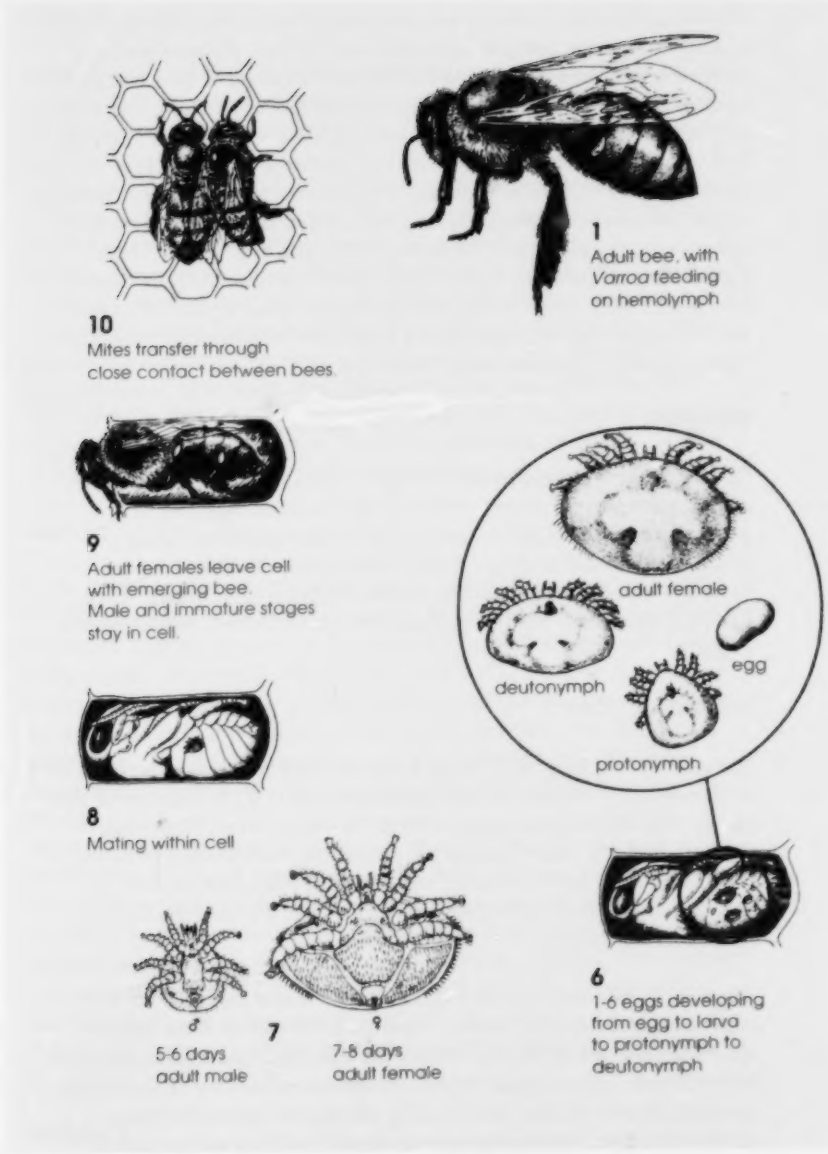


Figure 19. Life cycle of *Varroa jacobsoni*. (Courtesy of Roger A. Morse.)

Illustrated by B. Alexander

2
Mite enters cell with larva of 5 to 5-1/2 days.

3
Mite in bee food

4
Mite feeds on prepupa.

5
Female lays first egg 60 hours after cell capping. Female lays subsequent eggs at 30-hour intervals.

It is important to note that the bee-louse, Braula coeca, resembles *Varroa jacobsoni* in size and color. However, *Braula*, being an insect, has six legs that extend to the side (fig. 20). *Varroa*, an arachnid, has eight legs that extend forward (fig. 21).

When sampling, remember that the number and location of mites in a colony vary according to time of year. The number of mites is lowest in spring, increases during summer, and is highest in fall. During spring and summer, most mites are found on the brood (especially drone brood). In late fall and winter, most mites are attached to adult worker bees.

Methods of Examining Adult Honey Bees

For a sample of adult honey bees, 500 to 1000 bees should be collected. This can be done by brushing honey bees off the comb through a large-mouthed funnel (of paper or cardboard, etc.) into a container or by using a modified portable car vacuum cleaner. Individual honey bees can be examined with or without the aid of a hand lens or a dissecting microscope. When the mites are moving about on a bee, they are fairly easy to detect; but once they attach themselves between segments, they are difficult to find. Mites can be detected and collected by three methods, as follows:

Shaking Method

Varroa jacobsoni can be dislodged by shaking the bees in liquids such as hot water, alcohol, detergent solution, hexane,

Figure 20.
Braula coeca,
dorsal view.

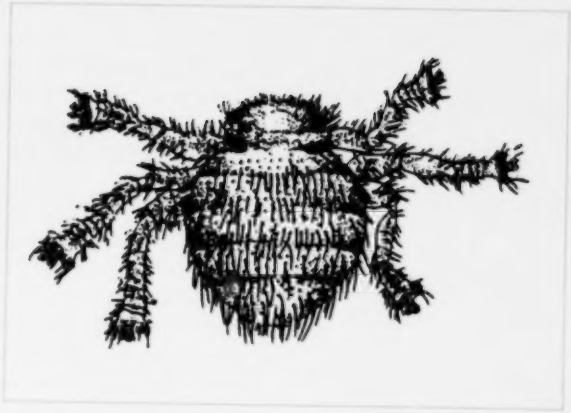


Figure 21.
Varroa
jacobsoni,
ventral view.

gasoline, or diesel fuel. We recommend 70% alcohol (ethyl or isopropyl) because some of the other materials are dangerous or difficult to use. The alcohol kills and preserves the bees for other purposes, such as examination for *Acarapis woodi*. De Jong et al. (1982a) found that hand-shaking bees in alcohol for 1 minute dislodged about 90% of the mites and that mechanical shaking on a rotary shaker for 30 minutes removed 100% of the mites. The mites are collected by passing the bees and alcohol through a wire screen (8- to 12-mesh) to remove the bees and then sieving the alcohol through a 50-mesh screen or cotton cloth. The screen or cloth is then examined for mites.

Ether Method

This technique is a rapid and efficient detection method in the field and avoids the handling, shipping, and time-consuming procedures associated with shaking adult bees in alcohol or other solvents. The bees (500-1,000) are collected in a jar and anesthetized with ether delivered from an aerosol can (this aerosol product is sold in auto-parts stores as an aid to start engines). A 1- to 2-second burst of material is adequate. The bees are then rotated in the jar for about 10 seconds. The majority of mites will have dislodged from their hosts and should be adhering to the inside wall of the jar. To complete the process, the bee sample is deposited on a white surface and spread around. This should cause any remaining mites to fall onto the white substrate. The bees should be examined immediately after the application of ether because the mites tend to stick to the bees if left in the jar for more than a few minutes. Alternatively, the bees can be left in the jar to which alcohol is added for laboratory shaking and preservation.

Heating Method

Live adult honey bees can be shaken into a wire-based cage and placed in an oven over white paper. The bees are heated for 10-15 minutes at 46°- 47°C. Then *Varroa jacobsoni*, if present, can be observed on the white paper (Crane 1978).

Methods of Examining Brood

To look for mites on brood, the pupae (preferably drone) are examined. *Varroa jacobsoni* can be easily seen against the white surface of worker or drone pupae after they are removed from their cells. It is suggested that a minimum of 100 drone pupae per colony be examined. The pupae can be collected by one of the following methods:

- The classic method of pupal collection is to uncap each cell and then remove the pupae with forceps or a hive tool.

- Groups of pupae can be quickly and easily removed from their cells by inserting a capping scratcher at an angle through the cappings and lifting the brood and cappings upward (Szabo 1989).

- With a long-bladed knife, the caps are sliced off an area of brood 4-6 square inches. The comb (frame) is then sharply jarred on a hard, flat, white surface such as a hive top. The brood will fall onto the white surface, and the mites can be easily observed.

• The brood comb can be incubated at 37°C, followed by examination of all the emerged bees and remaining brood.

Methods of Inspecting Hive Debris

Debris in a hive (such as wax particles, pollen, dead bees and brood, and mites) normally falls to the hive floor and is removed by house-cleaning bees during warm weather. This material can be collected and examined for the presence of *Varroa jacobsoni* as follows:

• The collection of hive debris can be facilitated by white construction paper on the hive floor. The paper is stapled under a wood (1/4-inch) and wire (8- to 12-mesh) frame, which protects the paper and debris from the bees. The paper is examined for mites, which can be easily seen against the white background. A magnifying glass or dissecting microscope can be helpful in locating the mites in the debris. Sticky boards or shelf paper (with the adhesive surface exposed) instead of construction paper will help hold the debris.

• The acaricides used to treat mite infestations can also be applied to bee colonies in combination with the paper method to detect *Varroa jacobsoni*. Apistan is currently approved and available for this purpose. After treatment, the mites drop to the paper and can be easily detected. It is important that the paper have a sticky surface (see previous paragraph) to hold any recovering mites.

• A flotation method can be used to examine debris for *Varroa jacobsoni*. Hive debris is placed in a jar or pan and covered with 98% alcohol. The mites float to the surface while the heavier debris sinks (Ritter and Ruttner 1980).

• Mites can sometimes be collected in dead bee or pollen traps attached to the colonies.

Tropilaelaps clareae

The distribution of *Tropilaelaps clareae* (fig. 22) is still restricted to Southeast Asia. These mites also parasitize adult bees and brood, and they have been reported to infest colonies infested with *Varroa jacobsoni* (Delfinado-Baker and Aggarwal 1987). Female mites are about 1 mm long and 0.6 mm wide; the male is slightly smaller. These mites are difficult to detect because of their small size and their brownish color, which blends perfectly with brood cappings and comb. *Tropilaelaps*

Figure 22.
Tropilaelaps clareae, ventral view.

clareae can be found by observing under a magnifying glass or a dissecting microscope a brood comb suspected of being infested. In the field, when the comb is hit on a light-colored surface, dislodged mites may be seen moving on that surface. The mites can be picked up with a fine brush moistened with alcohol.

Pests

Wax Moths

The greater wax moth, *Galleria mellonella* (fig. 23), is the most serious pest of honeycombs. Comb damage can also be caused by the lesser wax moth, *Achroia grisella* (fig. 23), and the Mediterranean flour moth, *Anagasta kuehniella*. These moths are an especially serious problem in tropical and subtropical climates, where warm temperatures favor the rapid development of the moths. Female greater wax moths lay their eggs in a cluster, usually in the cracks or between the wooden parts of the hive. The larvae of the moths are the destructive stage. They actually obtain nutrients from honey, castoff pupal skins, pollen, and other impurities found in the beeswax, but not the beeswax itself. Consequently, older combs are more likely to be infested than new combs or foundation.

Bee-louse (*Braula coeca*)

Braula coeca, or bee-louse (fig. 20), is actually not a louse but a wingless fly that feeds on honey. No detrimental effect on adult bees has been attributed to the bee-louse, but its larvae can damage the appearance of

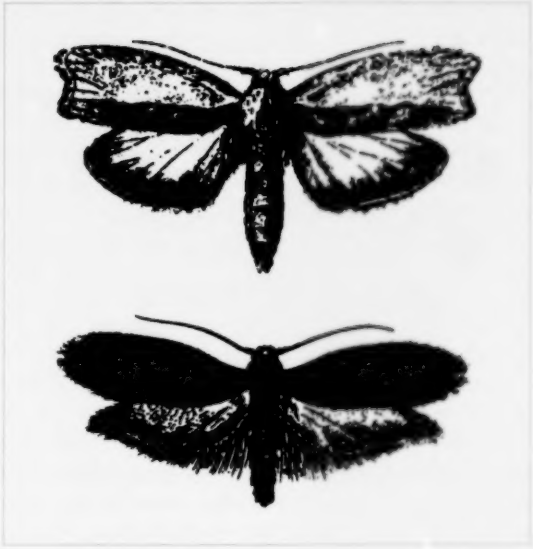


Figure 23. Greater wax moth, *Galleria mellonella* (top), and lesser wax moth, *Achroia grisella* (bottom) (not to scale). These species are easily distinguished from each other by shape of the wings and comparative sizes. Greater wax moth is about two times larger than lesser.

comb honey. Adult bee-lice can be found on adult workers and queens. It is important to note that *Braula coeca* resembles *Varroa jacobsoni* in size and color. However, Braula, being an insect, has six legs that extend to the side. *Varroa*, an arachnid, has eight legs that extend forward.

Melittiphis alvearius

Melittiphis alvearius is a little-known mite that is associated with adult honey bees but is not considered to be a pest. It is unlikely that *M. alvearius* would be confused with other mites found in honey bee colonies. The adult female mite is ovate, flattened dorsoventrally, 0.79 mm long and 0.68 mm wide, brown, and well sclerotized with numerous stout and spinelike setae. It is included here because it was found in California during a survey for *Varroa* and because of increasing reports on its distribution (Delfinado-Baker 1988).

Appendix A. Directions for Sending Diseased Brood and Adult Honey Bees for Diagnosis

The accuracy of the diagnosis of any bee disease depends on the condition of the sample. Mail the sample in a wooden or heavy cardboard box. The sample can be loosely wrapped in a paper bag, paper towel, newspaper, etc. Avoid wrappings such as *plastic bags*, *aluminum foil*, *waxed paper*, *tin*, or *glass* because they allow fungi to grow on the samples. Samples are accepted from anyone and in most cases are processed within one working day.

How to Address and Package Samples

- Send all samples to

  Bee Disease Diagnosis
  USDA, ARS, Bee Research Laboratory
  Building 476, BARC-East
  10300 Baltimore Avenue
  Beltsville, MD 20705-2350.

- A short description of the problem along with your name and address should accompany the sample. Also, your name and address should be plainly written on the top of the box.

Samples of Brood

- The sample of comb should be about 4 square inches.

- The sample should contain as much of the dead or discolored brood as possible.

- NO HONEY SHOULD BE PRESENT IN THE SAMPLE.

- If a comb cannot be sent, the probe used to conduct a ropiness test may contain enough material for tests. The probe can be wrapped in paper and sent to the laboratory in an envelope. However, this method is unsatisfactory for verification of sacbrood disease, which requires special antisera. We rely on gross symptoms for this diagnosis.

Samples of Adult Honey Bees

- Send at least *100 bees* in a sample.

- If possible, select bees that do not appear normal or that died recently. Decayed bees are not satisfactory for examination.

- Bees submitted for the identification of mites should be placed in 70% ethyl or methyl alcohol as soon as possible after collection. These samples should be in leak-proof containers.

Appendix B. Identification of Africanized Honey Bee

BRL Protocol for Identification of Africanized Honey Bee

FABIS, as described in the "Fast Africanized Bee Identification System (FABIS) Manual" (Sylvester and Rinderer 1987), will be used as a screening method to identify European bees. The full 26-character morphometric analysis will be conducted on all samples of honey bees not identified as European by FABIS. Therefore, when the Bee Research Laboratory (BRL) declares a sample Africanized, the identification is the result of the full 26-character morphometric analysis and is definitive.

Submission of Honey Bees to BRL for Identification of Subspecies

It is assumed that most honey bee samples submitted for identification of subspecies will be from Animal and Plant Health Inspection Service, Plant Protection and Quarantine (APHIS-PPQ). Samples will also be accepted from State regulatory personnel by prior arrangement. A sample of 100 bees should be submitted. Anyone submitting suspected Africanized honey bees should understand our priorities for identification and sample submission. They are as follows:

Priority No. 1: Swarms (colonies) anywhere in the United States involved in severe stinging incidents.

Priority No. 2: Any swarms (colonies) in the United States that have been pretested and identified as non-European by FABIS.

Honey bees submitted for identification should be placed in 70% ethyl or methyl alcohol as soon as possible after collection. Each sample should consist of at least 100 workers in leak-proof containers and be sent directly to

Bee Disease Diagnosis
USDA, ARS, Bee Research Laboratory
Building 476, BARC-East
10300 Baltimore Avenue
Beltsville, MD 20705-2350.

A short history of the problem along with the sender's name, address, and telephone number should accompany the sample. If results are urgently required, please advise the Laboratory by telephone (301-344-2205) of the shipment and send the sample via *Express Mail*.

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